J. Cell Sci. 73, 105–119 (1985)
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IMMUNOLOCALIZATION OF TISSUE INHIBITOR OF METALLOPROTEINASES (TIMP) IN HUMAN CELLS

CHARACTERIZATION AND USE OF A SPECIFIC ANTISERUM

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SUMMARY

A specific antiserum to pure human amniotic fluid metalloproteinase inhibitor (TIMP) was raised in a sheep. This antiserum was used to demonstrate: firstly, the immunological identity of the TIMP activities from amniotic fluid and culture medium of human foetal lung fibroblasts; and secondly, by indirect immunofluorescence, the secretion of TIMP by human foetal lung fibroblasts, chondrocytes, epithelial cells and smooth muscle cells. The phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate, was used to stimulate secretion of TIMP by human foetal lung fibroblasts and the ionophore monensin was used to demonstrate intracellular accumulation of TIMP in the Golgi apparatus of these cells. These results are discussed in relation to other inhibitors of collagenase reported in the literature, which are probably identical to TIMP.

INTRODUCTION

Inhibitors of collagenase have been described from culture media of connective tissues (Murphy, Cartwright, Sellers & Reynolds, 1977; Shinkai, Kawato, Hori & Nagai, 1977; Vater, Mainardi & Harris, 1979; Nolan, Ridge, Oronsky & Kerwar, 1980; Yasui, Hori & Nagai, 1981; Dean & Woessner, 1984) and cells (Nolan et al. 1978; Welgus *et al.* 1979; Kerwar *et al.* 1980; Pettigrew, Sodek, Wang & Brunette, 1980; Sapolsky *et al.* 1981; Macartney & Tschesche, 1983; Stricklin & Welgus, 1983; McGuire-Goldring *et al.* 1983), and in serum (Woolley, Roberts & Evanson, 1976; Woolley *et al.* 1978) and other body fluids (Bunning *et al.* 1984). The collagenase inhibitor from cultured rabbit bone was shown (Sellers, Murphy, Meikle & Reynolds, 1979) to inhibit two other neutral metalloproteinases, which degrade gelatin and proteoglycan. This inhibitor has been purified and characterized as a protein of molecular weight about 28 000 and was named TIMP, tissue inhibitor of metalloproteinases (Cawston *et al.* 1981). The inhibitor of collagenase from human amniotic fluid was found to have similar characteristics (Murphy, Cawston & Reynolds, 1981). It has been proposed that all connective tissues synthesize collagenase inhibitors to control the local activity of the enzyme (Reynolds, Murphy, Sellers & Cartwright, 1977; Murphy & Sellers, 1980) and it now seems likely that all the inhibitors are closely related to TIMP.

Key words: collagenase, proteinase inhibitor, TIMP, immunolocalization, monensin.

To help clarify the relationship between the various inhibitors and to elucidate their role in connective tissue metabolism we report here the preparation of a specific antiserum to the collagenase inhibitor derived from human amniotic fluid. This antiserum has been used to demonstrate the synthesis and secretion of TIMP-like inhibitors by human foetal lung fibroblasts and other human cell types. The phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), has been shown to stimulate synthesis of the collagenase inhibitor in HFL1 cells, and monensin to cause a build up of the 24000 M_r (less glycosylated) form in the cell layer (Murphy, Reynolds & Werb, 1985). In this work these drugs have been used to demonstrate, by indirect immunofluorescence, the intracellular accumulation of TIMP.

MATERIALS AND METHODS

Chemicals

Dulbecco's Modified Eagle's Medium (DMEM), trypsin–EDTA and foetal bovine serum (FBS) were from Flow Laboratories. All sera were heated at 56°C for 30 min before use, to inactivate complement. Monensin, agarose (electrophoresis grade) and 12-*O*-tetradecanoylphorbol-13-acetate were from Sigma. All other chemicals were reagent grade.

Cell cultures

Cultures of human foetal lung fibroblasts (HFL1) were given by Dr Eugene Martin (Department of Pathology, Rutgers Medical School, Piscataway, NJ, U.S.A.) and originated from the American Type Culture Collection, Rockville, MD, U.S.A. They were maintained in DMEM with 10% (v/v) FBS and sub-cultured with 0·25% trypsin–EDTA onto either glass coverslips, multiwell slides (Flow) or 8-well Labtek slides (Miles Laboratories, Ames Division) for immunolocalization experiments. TPA was added to some cultures (50 ng/ml, 48 h) from a 500 μg/ml stock solution in ethanol, stored at −20°C. Monensin (1 μM) was added to some cultures for 3 h immediately before fixation, from a 10 mM stock solution in ethanol.

Cultures of lst-passage human chondrocytes were given by Dr Mary Goldring, Arthritis Unit, Massachusetts General Hospital, Boston, MA, U.S.A.

Cultures of normal human dermal fibroblasts, human hypertrophic scar fibroblasts, dermal fibroblasts derived from a patient with Epidermolysis Bullosa distrophica recessive (EBdr), A549 epithelial cells and human smooth muscle cells were provided by Dr H. Paul Ehrlich, Shriners Burns Institute, 51 Blossom Street, Boston, MA, U.S.A. They were maintained in DMEM with 10% FBS and subcultured onto coverslips when required.

All the fibroblast cultures and the smooth muscle cells were plated at low density and used 4–6 days after plating whilst still subconfluent. The chondrocytes and A549 epithelial cells were plated at higher density (1×10^6 cells per 30 mm dish) to retain phenotype but were used 4–6 days after plating whilst still subconfluent.

Purification of TIMP

The inhibitors from human amniotic fluid and from fibroblast culture medium were purified as previously described (Murphy *et al.* 1981). Inhibitor activity was assessed also as described previously (Murphy *et al.* 1981) by inclusion with enzyme in the collagenase assay for 15–20 h incubations. Protein was estimated by the Fluram method (Weigele, DeBernado, Tengi & Leimgruber, 1972).

For the Sepharose absorption experiment about 0·35 μg TIMP was coupled to 1 ml cyanogen-bromide-activated Sepharose 4B (Pharmacia) according to the manufacturer's directions.

Preparation of antibody to TIMP

A 100 μg sample of purified amniotic fluid inhibitor was emulsified with an equal volume of Freund's complete adjuvant (Difco) and injected into two intramuscular sites in an adult Clun sheep. Two further intramuscular injections, each of 50 μg inhibitor in complete adjuvant, were given after 22 and 51 days. A preimmune bleed of 50 ml blood was taken from the jugular vein and further 400 ml bleeds taken on days 31, 34, 47, 58, 62 and 65. The blood was allowed to clot at room temperature, then placed at 4 °C overnight. Serum was removed by centrifugation and stored at −80 °C. Immunoglobulins (IgG) were prepared from the bleed with the highest titre (bleed no 6) by triple precipitations with 0·67 vol. 4 M-$(NH_4)_2SO_4$ (pH 7·0) at room temperature. The final precipitate was resuspended in 0·5 serum vol. phosphate-buffered saline (PBS) and dialysed against PBS at 4 °C. Immunoglobulins from pooled normal sheep serum were prepared in the same way (NSS-IgG). IgG concentrations were determined by absorbance (1 %) at 280 nm = 14 (Little & Donahue, 1968).

The ability of the antibody to inhibit TIMP from both amniotic fluid and fibroblast cultures was assayed using two purified metalloproteinases from rabbit bone, collagenase and proteoglycanase, in the standard assays (Murphy *et al.* 1981). Purified IgG was incubated with TIMP for 30 min at 37 °C before incubation with the enzymes in their respective assay systems.

Antibody specificity was checked by double immunodiffusion and rocket immunoelectrophoresis using standard methods (Ouchterlony & Nilsson, 1978). After washing, plates were stained in Coomassie Brilliant Blue, 1 mg/ml in ethanol/formic acid solvent and destained in solvent alone. Electrophoretic immunoblotting was performed esentially as described by Towbin, Staehelin & Gordon (1979). Crude and purified TIMP preparations were loaded after dissociation at 100 °C for 5 min by sodium dodecyl sulphate (SDS), and electrophoresed in a 12·5 % (w/v) polyacrylamide gradient slab gel. Transfer of proteins to nitrocellulose paper was effected in a Bio-Rad transblot cell (Bio-Rad Laboratories Ltd, Watford, Herts); 0·1 % SDS was added to the transfer buffer and the blocking buffer was 0·5 % casein, 0·05 % Tween 20, 0·9 % NaCl in 10 mM-Tris·HCl (pH 7·4). The nitrocellulose paper was washed in the Tris–NaCl buffer alone. The paper was incubated overnight in the sheep anti-human TIMP antiserum diluted 1 : 50 in blocking buffer, then washed and incubated for 2 h in a horseradish peroxidase-conjugated rabbit IgG to sheep IgG (Dako-immunoglobulins a/s, Denmark), diluted 1 : 200 in blocking buffer. Colour was developed with 4-chloro-1-naphthol (0·5 mg/ml) and 0·01 % H_2O_2 in washing buffer. Gels were stained for protein after transfer to confirm complete transfer.

Preparation of fluorescein-labelled pig Fab monomer against sheep Fab fragments (pig-FITC)

For immunocytochemical localization studies monovalent antibody fragments have major advantages over intact IgG, consequently we prepared a fluorescein-labelled pig Fab to sheep Fab monomer for use as a second antibody. Sheep Fab monomer was prepared from pooled normal sheep serum (2 % (w/v) pepsin digestion of IgG, 37 °C, 48 h) as described (Davies, Barrett & Hembry, 1978), checked on SDS/polyacrylamide gel electrophoresis (SDS/PAGE) for homogeneity, and double immunodiffusion for specificity against rabbit anti-whole sheep serum (Miles) and rabbit anti-sheep IgG (Miles) antibodies; 200 μg monovalent sheep Fab was then emulsified in an equal volume of Freund's complete adjuvant and injected into two intramuscular sites in an adult pig. Two further injections of 200 μg were given 24 and 42 days later. A test bleed of 20 ml was taken on day 48 and then the pig was killed and bled out. Serum was collected and IgG prepared as described above for the sheep antibody. Specificity of this pig anti-sheep Fab' antibody was checked by double immunodiffusion and immunoelectrophoresis and shown to react only with sheep Fab fragments.

Fab monomer of this pig antiserum was prepared as described for the sheep Fab, except that a 5 % pepsin digestion of the IgG for 30 h was used. This monomeric pig Fab was checked on SDS/PAGE for homogeneity and labelled with fluorescein isothiocyanate (BDH, Poole, Dorset) by the method of Thé & Feltkamp (1970). The final product (11·0 mg/ml, molar ratio 1·514) was stored at 4 °C and used diluted 1 : 300 in PBS.

Immunocytochemical methods

Capture experiments. To determine whether different cell types secrete TIMP, subconfluent cultures of cells were rinsed twice with warm DMEM, then fresh DMEM containing either normal sheep serum (NSS)-IgG (3 mg/ml) or antibody IgG (3 mg/ml) was added and the cells were incubated at 37°C in 5% CO_2/air for either 5 min, 20 min, 40 min, 1 h or 2 h. This medium was then replaced by DMEM with 10% (v/v) FBS and the incubation continued for a further 1 h in order to remove unbound sheep IgG. The cells were then washed briefly in PBS, fixed for 5 min in freshly prepared 4% paraformaldehyde (pH 7·4), and the fixative was removed with several washes of PBS. The coverslips were stained with the second antibody (pig-FITC) for 30 min at room temperature. The coverslips were then washed with PBS and mounted on slides in PBS–glycerol (1:9, v/v) mounting medium containing 1 mg/ml *p*-phenylenediamine (Johnson & Araujo, 1981). Cells were observed using either a Zeiss Photomicroscope III or a Zeiss IM35 inverted microscope, both fitted with standard FITC filters and epifluorescence. Photographs were taken on Kodak Ektachrome 400 film uprated to 1600 ASA.

To confirm that the sheep antibody was indeed specific for the human inhibitor, antibody IgG was allowed to react with TIMP inhibitor–Sepharose and the supernatant incubated with cultures of HFL1 cells for 2 h. Two polypropylene minicentrifuge tubes (Alpha Labs, Hampshire, U.K.) containing either 100 μl pure TIMP–Sepharose 4B (see Materials and Methods) or 100 μl Sepharose 4B were centrifuged at 10 000 ***g*** for 5 min and the pellets washed three times in 0·9% NaCl. Both pellets were then resuspended in 60 μl DMEM, 90 μg antibody IgG was added to each and the tubes incubated for 30 min at 4°C to allow antibody–antigen binding to take place. The tubes were then centrifuged, the supernatants removed and assayed for anti-TIMP activity. No activity could be detected in the antibody absorbed with TIMP–Sepharose; full activity was present in the antibody absorbed with Sepharose 4B alone. HFL1 cells were grown on multiwell slides in DMEM with 10% FBS, and rinsed briefly in warmed DMEM. DMEM containing either antibody IgG (30 μg/20 μl), antibody absorbed on TIMP–Sepharose 4B, or antibody IgG absorbed on Sepharose 4B, was added to duplicate wells on the multiwell slide, 20 μl/well. The slide was then incubated at 37°C for 2 h followed by a further 1 h incubation with 20 μl DMEM containing 10% FBS in all the wells. Care was taken to dry the plastic coating of the slides between the wells thoroughly to prevent cross-contamination of the different media, but to prevent drying of the media during incubation by provision of a very humid atmosphere. The slides were then rinsed, fixed and stained as described above, using 20 μl pig-FITC in each well.

Intracellular localization. For intracellular localization of TIMP, cells were rinsed briefly with PBS, fixed as above, permeabilized with 0·1% Triton X-100 in PBS for 5 min, then washed repeatedly. The cells were then incubated for 30 min at room temperature with either antiserum IgG (50 μg/ml) or NSS-IgG (50 μg/ml). They were then washed and treated with the second antibody pig-FITC as above.

RESULTS

Anti-TIMP antibody inhibition and specificity

The ability of the anti-TIMP IgG to inhibit the activity of TIMP against purified metalloproteinases was studied. The inhibitor can prevent the activity of a number of these proteinases derived from connective tissues (Sellers *et al.* 1979; Murphy *et al.* 1981). Using collagenase and proteoglycanase purified from rabbit bone, it was shown that their inhibition by TIMP, purified from either amniotic fluid or fibroblast culture medium, was prevented by pretreatment of TIMP with the antibody (Fig. 1).

The specificity of the antiserum was examined by three different methods: immunodiffusion, rocket immunoelectrophoresis and electroimmunoblotting. In

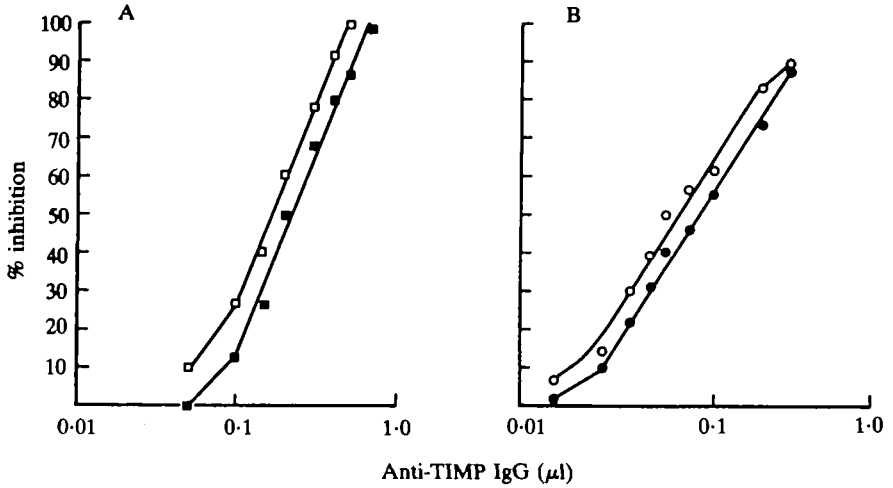
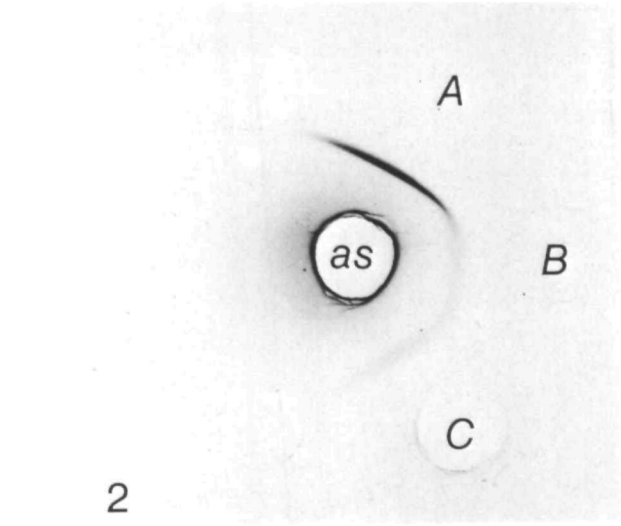


Fig. 1. The inhibition of TIMP activity against two metalloproteinases by the anti-TIMP antiserum. Varying amounts of purified anti-TIMP IgG (14 mg/ml) were pre-incubated with purified preparations of TIMP from either amniotic fluid or fibroblast conditioned medium, then assayed for residual inhibitory activity against proteoglycanase (A) and collagenase (B) in their respective standard assays. (□) 0·024 unit of amniotic fluid inhibitor anti-proteoglycanase activity; (■) 0·026 unit of fibroblast inhibitor anti-proteoglycanase activity; (○) 0·051 unit of amniotic fluid inhibitor anti-collagenase activity; (●) 0·044 unit of fibroblast inhibitor anti-collagenase activity (1 unit of inhibitor will inhibit 2 units of proteinase by 50%; approximately 4 units of proteoglycanase inhibitory activity is equivalent to 1 unit of collagenase inhibitory activity).

immunodiffusion a single line of identity formed when either the IgG or the whole serum was run against the pure inhibitor from amniotic fluid, the material that bound to concanavalin A (ConA)–Sepharose during the purification of the amniotic fluid inhibitor, and concentrated (100-fold) medium from cultures of HFL1 cells (Fig. 2). A single precipitin line was also formed when materials from various stages of the purification of the amniotic fluid inhibitor were run on rocket immunoelectrophoresis (Fig. 3). It is noteworthy that the form of the inhibitor that did not bind to ConA-Sepharose (Murphy *et al.* 1981) also reacted with the antiserum (Fig. 3). The binding and non-binding forms of the inhibitor showed a reaction of complete identity when diffused against the anti-TMP antiserum (results not shown). Since it is likely that the latter is a non-glycosylated form of TIMP it appears that the antiserum is, at least in part, raised to antigenic determinants on the protein portion of the molecule. A single band was also detected when crude and pure TIMP from both amniotic fluid and medium from HFL1 cells was electroblotted onto nitrocellulose paper (Fig. 4). These data demonstrate that the antiserum is specific for TIMP, and that the form of TIMP from human amniotic fluid is immunologically identical to the inhibitor produced by HFL1 cells. Concentrated human lung washings and concentrated medium from cultures of human heart valve, meningioma and tendon also gave a single line of identity on Ouchterlony gel diffusion (data not shown).



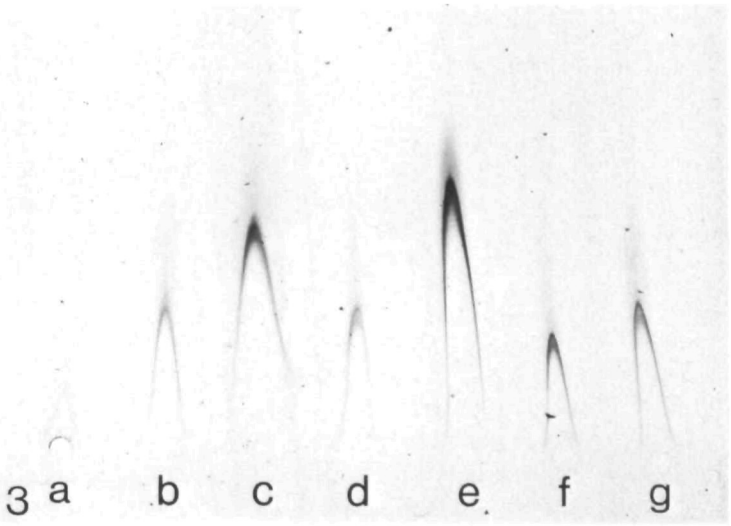


Fig. 2. Immunodiffusion of sheep anti-TIMP antiserum against crude and purified TIMP from amniotic fluid and HFL1 cells. Sheep anti-TIMP antiserum (*as*, 5 μl) in the central well was allowed to diffuse for 24 h against: *A*, crude amniotic fluid inhibitor bound to ConA–Sepharose, 10 μl; *B*, pure amniotic fluid inhibitor, 5 μl; *C*, 100 × concentrated medium from HFL1 cells, 5 μl. The plate was then washed and stained with Coomassie Brilliant Blue.

Fig. 3. Rocket immunoelectrophoresis of material from various stages of the purification of the amniotic fluid inhibitor (Murphy *et al.* 1981); 0·6% sheep anti-TIMP antiserum was added to the gel and 3 μl samples were electrophoresed at 20 mA, 300 V, overnight. Lanes a, crude ammonium sulphate fraction; b, ConA-binding pool; c, ConA non-binding pool; d, DEAE-Sepharose pool; e, heparin–Sepharose pool, 1·24 units; f, heparin–Sepharose pool, 0·53 unit; g, Zn^{2+}-iminodiacetic acid–Sepharose pool. The plate was then washed and stained with Coomassie Brilliant Blue.

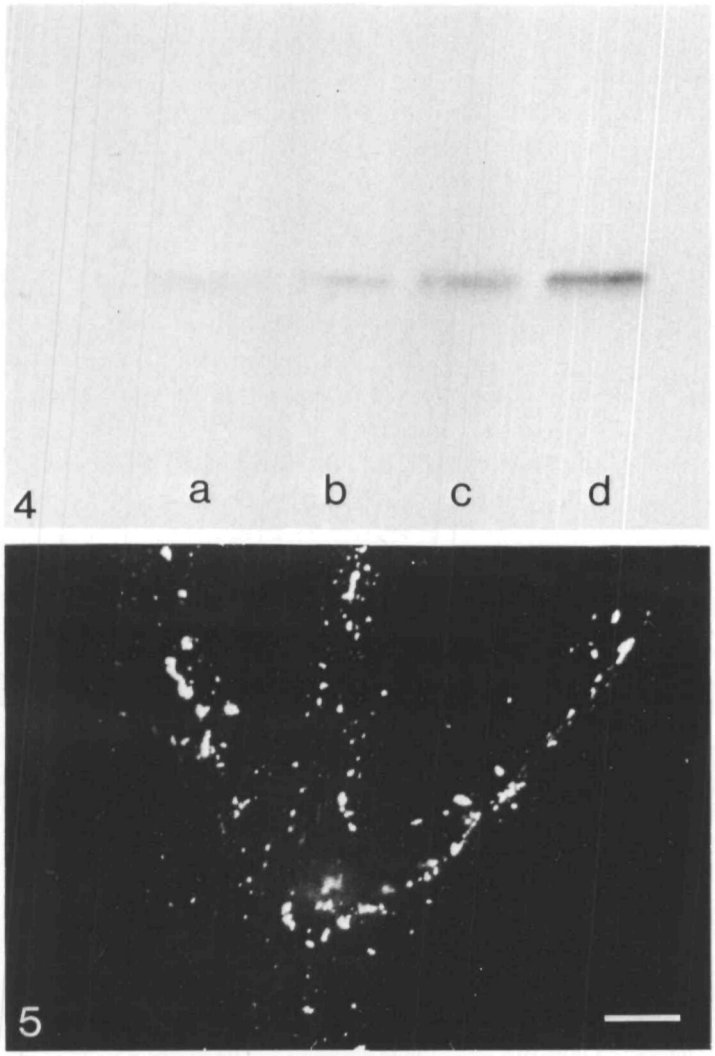


Fig. 4. Electroblot of pure and crude TIMP from amniotic fluid and HFL1 cells. Samples were loaded after dissociation by boiling with SDS and electrophoresed in a 12·5% polyacrylamide gradient slab gel. Proteins were transferred to nitrocellulose paper, which was then incubated overnight in sheep anti-TIMP antiserum, followed by horseradish peroxidase-conjugated rabbit anti-sheep IgG. Colour was developed with 4-chloro-1-naphthol. Lanes a, crude HFL1 medium, 10 μl; b, pure HFL1 inhibitor, 5 μl; c, pure amniotic fluid inhibitor, 5 μl; d, crude ConA-binding amniotic fluid material, 10 μl.

Fig. 5. Extracellular immunoprecipitation around an HFL1 cell. HFL1 cells were incubated with sheep anti-TIMP antiserum for 2 h, followed by 1 h with DMEM+10% FBS. Cells were then fixed and stained with pig-FITC. Bar, 30 μm.

Immunolocalization of TIMP

Capture experiments. The capture of secreted proteins by polyclonal antibody, as they are released by the cell to an extracellular site with the formation of extracellular immunoprecipitates, was first demonstrated with the lysosomal proteinase, Cathepsin D (Poole, Hembry & Dingle, 1974). This is a useful method for demonstrating protein secretion from living cells, and has been modified to demonstrate TIMP release from HFL1 fibroblasts in monolayer culture. When HFL1 cells were incubated at 37°C in the presence of either the antiserum IgG (3 mg/ml) or whole immune serum in capture experiments, extracellular immunoprecipitates were formed (Fig. 5). No staining was visible after 5 min of culture, indicating that the cultures contained no residual TIMP from the previous culture period. After 20 min of incubation in the presence of the antiserum, very small immunoprecipitates could be seen associated with a few cells. Most cells were negative. At 40 min and 1 h the immunoprecipitates were seen to be associated with an increasing number of cells, particularly around the ruffling edge of the fibroblasts. By 2 h the immunoprecipitation had intensified and almost all cells had some staining associated with them. No immunoprecipitation was seen when cells were cultured for 2 h with either NSS-IgG or the preimmune sheep serum (5 % in DMEM). No intracellular staining was visible at any of the above times with either antiserum IgG or NSS-IgG.

In absorption experiments, to confirm the specificity of the antiserum, cells incubated for 2 h with antibody IgG absorbed on Sepharose 4B showed intense immunoprecipitation. However, cells incubated for 2 h with antibody absorbed on TIMP–Sepharose 4B gave no immunoprecipitation, showing that the TIMP–Sepharose had removed all the specific antibody, as indicated by the assay results.

To investigate which other human cell types also produced TIMP, a variety of cells were cultured for 2 h with either antiserum IgG or NSS-IgG (see Materials and Methods, *Capture experiments*). No staining was ever seen in the NSS-IgG-treated cells of any cell type. All antiserum-treated cultures showed extracellular immunoprecipitation clearly associated with some but not all cells. In the fibroblast cultures an estimated 90 % of cells showed some immuno-staining after the 2 h culture period, whereas only about 20 % of chondrocytes had associated immunoprecipi-

Fig. 6. A. Perinuclear immunofluorescence (Golgi apparatus) in unstimulated HFL1 cells. HFL1 cells were fixed, then stained with sheep anti-TIMP IgG followed by pig-FITC. Arrow shows weak but positive staining. Bar, 10 μm. B. Unstimulated HFL1 cells incubated with monensin for 3 h before staining with sheep anti-TIMP IgG. HFL1 cells were incubated in monensin (1 μM, 3 h), then fixed and stained as in A. Golgi staining is intense and fluorescent cytoplasmic granules are present (arrows). Bar, 10 μm. C. TPA-treated HFL1 cells incubated with monensin for 3 h before staining with sheep anti-TIMP IgG. HFL1 cells were stimulated with TPA (48 h, 50 ng/ml) and monensin was added for the last 3 h before fixation. Cells were then stained as for A. Golgi staining is brilliant and vacuolated, and some cytoplasmic staining is visible (arrow). Bar, 10 μm. D. TPA-treated HFL1 cells incubated with monensin for 3 h before staining with NSS-IgG. HFL1 cells were treated as in C, but stained with NSS-IgG. No staining is visible. Bar, 10 μm.

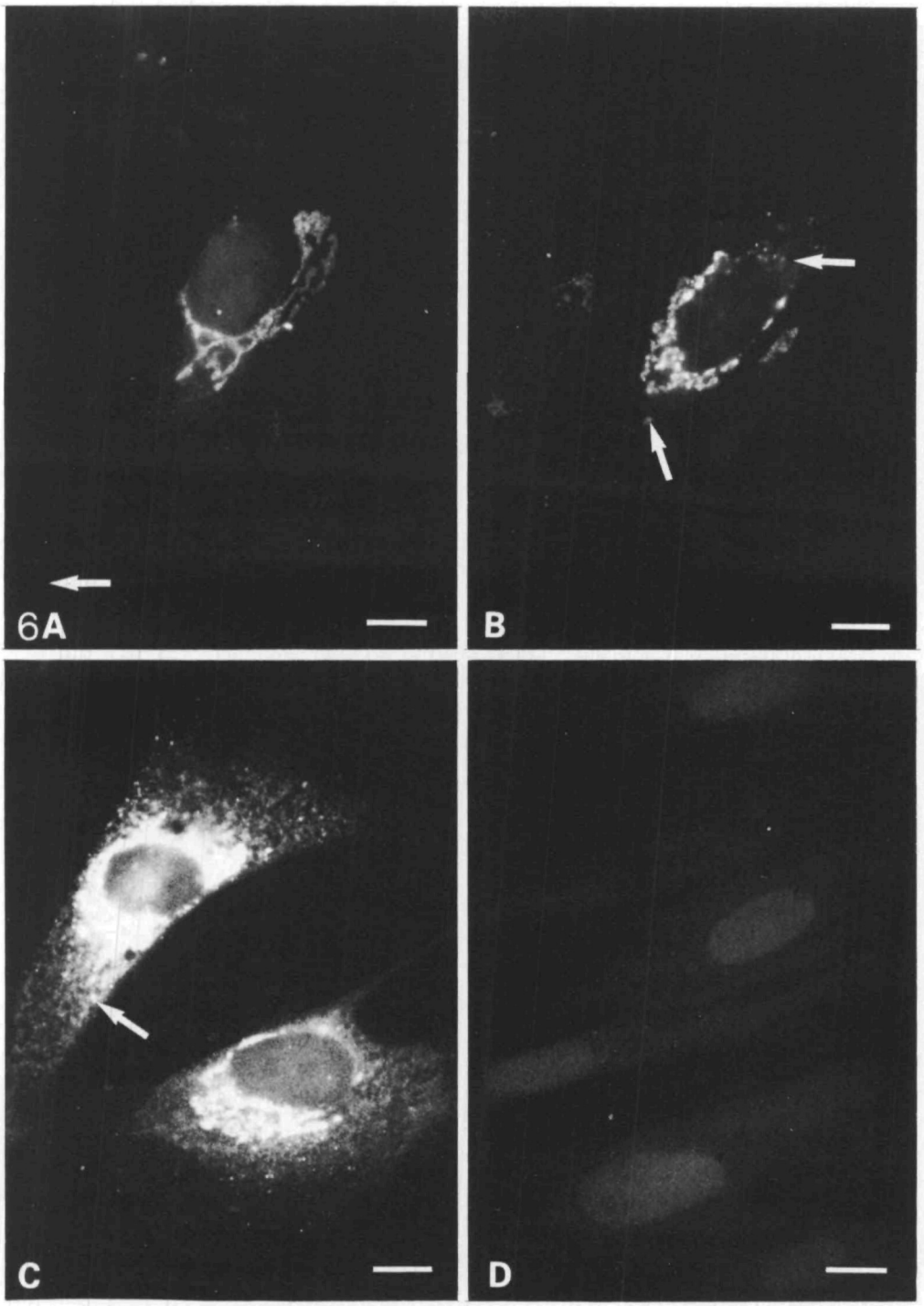


Fig. 6

tation. The A549 epithelial cells showed the least number of active cells. In 2 h cultures of human smooth muscle cells, almost all had immunoprecipitation around them.

Intracellular localization. As some HFL1 cells were surrounded by considerable immunoprecipitation after a 2 h culture period, suggesting significant secretion of TIMP, intracellular localization was attempted (see Materials and Methods) to locate the source of the inhibitor. No staining was seen within cells treated with NSS-IgG, and cells treated with antiserum IgG showed no extracellular staining (confirming lack of binding of antiserum IgG to matrix components, and lack of inhibitor entrapped within the matrix). However, some antiserum-treated cells showed bright fluorescence in the perinuclear region, probably the Golgi apparatus: approximately 30 % of cells had some perinuclear fluorescence, although it was often rather weak (see arrow in Fig. 6A). The monovalent ionophore monensin has been shown to interfere with the processing and secretion of proteins from human fibroblasts (Ledger, Uchida & Tanzer, 1980). When monensin was included in the culture medium of HFL1 cells for 3 h (see Materials and Methods) before intracellular staining, the perinuclear fluorescence became much more prominent, often vacuolated and (Fig. 6B) visible in a greater number of cells. Some cells also contained fluorescent cytoplasmic granules (see arrows in Fig. 6B). However, many cells, often in contiguous areas, showed *no* intracellular staining, whereas in other areas nearly all cells were stained, as in Fig. 6B.

The addition of TPA to the culture medium of HFL1 cells has been shown to increase the production of TIMP 3- to 10-fold (Murphy, Reynolds & Werb, 1985) over a 48–72 h period. When this agent was used, in conjunction with monensin treatment for the last 3 h of the culture period (see Materials and Methods), intense perinuclear staining was seen in the majority of cells stained with antiserum IgG. Some cells also had bright cytoplasmic fluorescence (Fig. 6C). Cells treated similarly, but stained with NSS-IgG, had no fluorescence (Fig. 6D), either cytoplasmic or perinuclear.

DISCUSSION

TIMP, the tissue inhibitor of metalloproteinases, was originally characterized as a protein (M_r 28000) from rabbit bone culture medium (Cawston *et al.* 1981) and is found in most tissues and cells in culture (Reynolds, Bunning, Cawston & Murphy, 1981). It can form a tight complex with either active collagenase or active proteoglycanase, which differs from the latent enzyme forms (Cawston *et al.* 1983) and may represent the final 'fail-safe' mechanism to control the extracellular activity of these enzymes. The inhibitor from human amniotic fluid was found to have characteristics similar to those from rabbit bone cultures (Murphy *et al.* 1981). In this paper we report the preparation and use of a specific antiserum to the human amniotic fluid collagenase inhibitor. We have demonstrated: firstly, the immunological identity of the TIMP activities from amniotic fluid and culture medium of human foetal lung fibroblasts; and secondly, by indirect immunofluorescence, the

secretion of TIMP by human foetal lung fibroblasts. This paper records the first visual demonstration of TIMP secretion by living cells and TIMP has also been localized intracellularly, possibly on the Golgi apparatus of actively secreting cells. We have also shown that other human cell types, chondrocytes, epithelial cells, smooth muscle cells and various fibroblasts cell lines, also secrete immunoprecipitable TIMP and the relationship between this and other inhibitors of collagenase, reported by other workers, is discussed below.

Culture media from monolayer cultures of human articular chondrocytes were found to contain a collagenase inhibitor of apparent molecular weight about 30 000 (McGuire-Goldring *et al.* 1983) and bovine articular chondrocyte cultures also produce a metalloproteinase inhibitor (Morales, Kuettner, Howell & Woessner, 1983) apparently similar to TIMP. We have confirmed that human articular chondrocytes in monolayer culture are able to secrete immunoprecipitable TIMP. No attempt at quantitation was made in these experiments. The chondrocytes and A549 epithelial cells (see below) were plated at high density to retain their phenotype, and showed less immunoprecipitation than HFL1 fibroblasts, perhaps because the cultures were approaching confluence. The development of an ELISA assay for human TIMP will facilitate this type of quantitation.

Pettigrew *et al.* (1980) described an inhibitor of collagenase from porcine periodontal ligament epithelial cell culture medium, which also inhibits non-specific gelatinase. Our cultures of human A549 epithelial cells also produced immunoprecipitable TIMP, confirming that epithelial cells are also able to make this inhibitor. Interestingly, Welgus & Stricklin (1983) reported that monolayer cultures of human epidermal keratinocytes produced a collagenase inhibitor (see below) but at such extremely low levels that they could not rule out trace contamination of low-passage cultures with dermal fibroblasts. In our cultures, examined by indirect immunofluorescence, the epidermal cell was clearly seen to be the secreting-cell type.

We have also demonstrated that human fibroblast cultures derived from normal skin, hypertrophic scar and dermal fibroblasts derived from a patient with Epidermolysis Bullosa distrophica recessive (EBdr) were able to release a protein immunologically precipitable with the anti-TIMP antiserum. Human skin fibroblasts in monolayer culture have been reported to synthesize a specific inhibitor of active collagenase of similar molecular weight to TIMP, 28 500 (Welgus *et al.* 1979; Stricklin & Welgus, 1983). An antiserum to this inhibitor has been described (Welgus & Stricklin, 1983), although the specificity of this antiserum was analysed only by Ouchterlony gel diffusion. These authors reported a line of identity on Ouchterlony gel diffusion between culture media from various human fibroblasts, foetal osteoblasts, uterine smooth muscle cells, fibrosarcoma cells and explants of tendon and articular cartilage. Furthermore, immunoreactive protein was found in serum and amniotic fluid. The properties of this inhibitor and its ubiquitous occurrence suggest that it is probably identical to TIMP and that its specificity for collagenase should be re-examined to include other metalloproteinases.

A collagenase inhibitor from porcine aorta smooth muscle cells has been reported

(Nolan *et al.* 1978), partially purified (Kerwar *et al.* 1980), and found to be a glycoprotein of apparent molecular weight 25 000. In our experiments human smooth muscle cells were shown to produce TIMP, and the relationship between these two inhibitors should be clarified further.

Intracellular immunolocalization of TIMP resulted in fluorescence of a discrete perinuclear lamellar structure. This was taken to be the Golgi apparatus, since it became vacuolated on monensin treatment. Ledger *et al.* (1980) described the effects of monensin on human fibroblasts in culture and showed, also by immunofluorescence microscopy, the intracellular accumulation of procollagen and fibronectin initially in the juxta-nuclear region and subsequently also in peripheral regions. Other workers have raised antibodies to specific Golgi proteins (Louvard, Reggio & Warren, 1982; Lin & Queally, 1982), which gave a distinct perinuclear fluorescence similar to the one described here. Further work using electron microscopic immunolocalization techniques should confirm this Golgi localization and further define the granular and cytoplasmic staining seen in some cells treated with TPA and monensin. The usefulness of monensin in these studies suggests that the procedure of increasing intracellular accumulation of an antigen might have much wider application. We are currently using this method to localize collagenase and TIMP in samples of synovium and cartilage from patients with rheumatoid arthritis, and in human tumours.

The phorbol ester, TPA, has been shown to stimulate synthesis of TIMP in HFL1 cells by 3- to 10-fold (Murphy *et al.* 1985). This increase was clearly visible in these experiments using indirect immunofluorescence. In capture experiments the amount of immunoprecipitation per cell increased as did the number of active cells (data not shown), and more cells showed intracellular staining in TPA-treated cultures than in unstimulated cultures. This result, and accumulation of TIMP in the Golgi apparatus of monensin-treated cultures, is consistent with the idea of continuous synthesis of TIMP that is stimulatable by the treatment of the cells with TPA. The mechanism of TPA stimulation is not known but phorbol esters can activate the phospholipid and Ca^{2+}-dependent protein kinase, kinase C, probably due to its structural similarity to diacylglycerol the endogenous kinase C activator. A number of subsequent effects on the metabolism of fibroblasts have been described (Gilmore & Martin, 1983), including the stimulation of collagenase secretion (Brinkerhoff, McMillan, Fahey & Harris, 1979).

A small number of cells in each culture showed neither extracellular precipitation nor intracellular fluorescence following treatment with TPA and monensin. The reason for these 'inactive' cells is not known: a longer incubation in the antiserum or monensin might perhaps render them 'active'. The cell cultures used in this study were not synchronized so it is possible that HFL1 cells might only synthesize TIMP during certain periods of the cell cycle. Alternatively, these cultures are known to make both proteoglycanase and collagenase (G. Murphy, R. Hembry & Z. Werb, unpublished observations) and experiments are in progress to show whether these cells make *either* enzymes *or* TIMP, or whether (and under what conditions) they make both at once. Deliberate manipulation of this balance between metallopro-

teinases and their inhibitors will then enable us first to define and then ultimately to control the extent of connective tissue destruction.

We thank the Medical Research Council for financial assistance and the Institute of Animal Physiology, Babraham for provision of sheep and pigs. We thank our colleagues, particularly Dr John T. Dingle, for their interest and encouragement and we also thank Dr Zena Werb for helpful suggestions.

Some of this work was done at the Shriners Burns Institute, Boston, U.S.A. and one of us (R.M.H.) thanks Dr H. Paul Ehrlich for encouragement, cell culture facilities and a year's financial support.

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(Received 25 June 1984 – Accepted 23 July 1984)